QTL Mapping of Winter Hardiness Genes in Lentil

A. Kahraman, I. Kusmenoglu, N. Aydin, A. Aydogan, W. Erskine, and F. J. Muehlbauer*

ABSTRACT

Lentil (*Lens culinaris* Medik.) germplasm with sufficient winter hardiness to survive most winters in cold northern areas is available. However, the use of that germplasm in breeding programs is hampered by variable winter conditions that make field evaluations needed for effective breeding and selection difficult. Our objectives were to gain additional information on the genetics of winter hardiness in lentil by QTL analysis and to identify markers for use in marker-assisted selection. A total of 106 F_6 derived recombinant inbred lines (RILs) from the cross WA8649090/Precoz were evaluated for winter survival in the field at Pullman, WA, USA, Haymana, Turkey, and Sivas, Turkey, in a randomized complete block design with three replications over 3 yr. Winter survival was based on plant stand counts before and after winter. In addition, winter injury was monitored at Pullman during the 1998-1999 winter season. Mean survival of the RILs was 49.7, 5.3, and 89.5% at Haymana in 1997-1998, at Pullman in 1998-1999, and at Haymana in 1999-2000, respectively. For QTL analysis of winter survival, three QTL were detected at Haymana in 1997-1998, one QTL was detected at Pullman in 1998-1999, and three QTL were identified at Haymana in 1999-2000. Only one of the QTL was common to all environments. For winter injury scores at Pullman in 1999, four QTL were identified that influenced winter survival. Overall results indicated that winter hardiness is influenced by several genes and the cumulative effects of winter stress.

Susceptibility to cold temperature limits the use of lentil as a fall-sown winter annual crop in temperate highland areas of the world. Germplasm has been identified with good winter hardiness (Erskine et al., 1981; Spaeth and Muehlbauer, 1991), which makes it possible to breed lentil cultivars that can be fall seeded. However, the genetics of winter hardiness in lentil is not well understood. Identifying genetic factors that contribute to winter survival is critical to effective breeding for winter hardiness in lentil.

Genetic studies on winter hardiness of lentil, using recombinant inbred line populations, indicated that the trait is controlled by several genes (Kahraman et al., 2003). Winter hardiness in pea (*Pisum sativum* L.) is reportedly controlled by dominant genes (Cousin et al., 1985) and additive genes (Auld et al., 1983). Three or four genes appeared to be responsible for winter hardiness in pea (Liesenfeld et al., 1986). Cold tolerance in chickpea (*Cicer arietinum* L.) is reportedly controlled by at least five genes with tolerance dominant over susceptibility (Malhotra and Singh, 1990).

One of the major problems in characterizing the genetic control of winter hardiness is inconsistency of field and freezing tests. Assessing winter hardiness in the field can be affected by numerous environmental factors including cold temperatures, freeze-thaw cycles, water logging, ice encasement, and diseases (Dexter, 1956; Lewitt, 1980; Blum, 1988). The complexity of winter hardiness is dependent on developmentally regulated processes such as the ability to acclimate to low and freezing temperatures and the ability to alter physiologically complex pathways (Palta et al. 1997). For example, in cereals the expression of winter hardiness depends on a number of interacting factors including vernalization requirement, response to changing photoperiod, and tolerance to low temperatures (Pan et al., 1994). In legumes, it is not known whether vernalization and photoperiod sensitivity are required for development of winter hardiness.

Improving winter hardiness on a phenotypic basis is difficult because the trait is complex and strongly affected by environmental factors. Moreover, screening for winter hardiness is hampered by the existence of genotype × environment interactions. DNA markers closely linked to the winter hardiness genes represent a promising selection tool.

With molecular marker technology, it may be possible to elucidate the genetics of winter hardiness in lentil. Genetic studies of winter hardiness using molecular techniques have been reported in various crops including barley (*Hordeum vulgare* L.) (Hayes et al., 1993; Pan et al., 1994), oil seed brassica (*Brassica napus* L.) (Teutonico et al., 1995), and alfalfa (*Medicago sativa* L.) (Brouwer et al., 2000). Molecular marker analysis of winter hardiness in wheat (*Triticum aestivum* L.) showed that different QTL controlled vernalization requirement and frost tolerance (Galiba et al., 1995), while QTL controlling vernalization and freezing tolerance in oil seed rape were mapped to the same genomic region (Teutonico et al., 1995).

Genetic linkage maps have been developed for various lentil populations (Havey and Muehlbauer, 1989; Muehlbauer et al., 1989; Tahir, 1990; Eujayl et al., 1998), but none included the genes for winter hardiness. The use of molecular markers to map genomic regions controlling winter hardiness and related traits would improve our understanding of the genetic control of these traits. A RIL population from a winter hardy × non-

A. Kahraman, Harran Universitesi Ziraat Fakultesi Tarla Bitkileri Bolumu, Sanliurfa, Turkey 63040; I. Kusmenoglu, Ihracatci Birlikleri Tohumculuk ve Arastirma San. Ve Tic. A.S. Ergazi Mah. Koyici Serpmeleri No. 4 Batikent/Ankara, Turkey; N. Aydin, A. Aydogan, Ankara Tarla Bitkileri Merkezi Arastirma Ens. Mud. PK 226 Ulus, Ankara, Turkey; W. Erskine, ICARDA, International Center for Agricultural Research in the Dry Areas, P.O. Box 5466, Aleppo, Syria; F.J. Muehlbauer, USDA-ARS and the Department of Crop and Soil Sciences, 303W Johnson Hall, Washington State University, Pullman, WA 99164-6434, USA. Received 15 Oct. 2002. *Corresponding author (muehlbau@wsu.edu).

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677 S. Segoe Rd., Madison, WI 53711 USA

Abbreviations: AFLP, amplified fragment length polymorphism; ISSR, inter simple sequence repeats; QTL, quantitative trait loci; RAPD, random amplified polymorphic DNA; RIL, recombinant inbred line; WH, winter hardiness.

hardy cross (WA8649090/Precoz) could be useful for QTL analysis of winter hardiness.

MATERIAL AND METHODS

An F_2 population from the cross of WA8649090 (winter hardy)/'Precoz' (non-hardy) was advanced by single seed descent to produce 106 F_6 derived recombinant inbred lines (RILs). The 106 RILs were then planted in the field at two locations in the fall of 1997, at three locations in the fall of 1998, and at three locations in the fall of 1999. The locations were Pullman, WA, USA, and Haymana, Turkey, in 1997, while in 1998 and 1999 the locations were Pullman, WA, USA, and Haymana and Sivas, Turkey. Soil types were fine-silty, mixed mesic Pachic Ultic Haploxerollos at Pullman; silty-clay at Haymana; and clay-loam at Sivas. The field plots were arranged in a randomized complete block design with three replications. Checks included the original parental lines and 'Brewer' as the non-hardy check. Plots were single rows 1 m long with an average of 30 to 40 plants in each row. Rows were spaced about 0.3 m apart. In the first year of field evaluations, experiments at Pullman were seeded in a conventionally tilled field on 15 Oct. 1997 while in the second and third years of field evaluations, the seeds were planted on 5 Oct. 1998 and 4 Oct. 1999 in minimally tilled fields with barley stubble. At Haymana and Sivas locations, planting was performed in a conventionally tilled field. Planting dates at Haymana were 23, 9, and 18 Oct. 1997, 1998, and 1999, respectively, while at Sivas, the planting dates were 12 and 20 Oct. 1998 and 1999, respectively.

Winter survival was based on plant stand counts recorded after seedling establishment in the fall and after regrowth in the spring. In the 1998-1999 field test at Pullman, winter injury to the above ground plant parts was scored throughout the winter. Winter injury to the plants was assessed by visually observing the amount of necrosis, withering, and wilting in each row. Intensity of the winter injury was rated as percentage damage on a plot basis where 0 to 10% indicated no damage or only leaf tips slightly damaged, while 90 to 100% indicated all plants in a row withered with no possibility of recovery. Scoring for injury was assessed at approximately 1-mo intervals (3 Jan., 9 Feb., 6 Mar., and 3 Apr. 1999). Analysis of variance was conducted separately for each environment using SAS PROC MIXED and PROC GLM procedures (SAS, 1996).

DNA Isolation and PCR Procedures

DNA was isolated from each RIL and the parents by taking leaf samples (1.5–2.0 g) before flowering and placing the samples in liquid nitrogen. The samples were stored at −80°C. Total genomic DNA was extracted by the miniprep method of Doyle and Doyle (1987) with some modifications as described in Simon and Muehlbauer (1997).

The protocols for RAPD (random amplified polymorphic DNA) (Williams et al., 1990) and ISSR (inter simple sequence repeats) analyses were performed on the basis of established procedures (Simon and Muehlbauer, 1997; Ratnaparkhe et al., 1998). A total of 800 decamer RAPD primers (UBC 1 to 800) and 100 ISSR primers with 15 to 23 nucleotides in length (UBC 801 to 900) were obtained from The Biotechnology Laboratory, University of British Columbia, Vancouver, BC, Canada. Also, 70 additional RAPD primers (CS 1 to 70) were obtained from Genosys Biotechnology Inc. (The Woodlands, TX). These primers were used to screen the parental lines for polymorphism. Primers that produced polymorphic PCR products were used for linkage mapping. Polymerase chain reactions were performed with Perkin Elmer 9600 and 9700 thermocyclers (Perkin-Elmer, Norwalk, CT).

Table 1. AFLP primer combinations used to screen the lentil parental lines for polymorphism.

Primer combinations	Nomenclature for primer combinations
***Eco*RI/*Mse*I +3 primers**	
act-ctt	E1M2
act-caa	E1M3
act-cag	E1M4
aca-ctg	E2M1
aca-ctt	E2M2
aca-cag	E2M4
agc-ctg	E3M1
agc-caa	E3M3
agc-cag	E3M4
***Pst*I/*Mse*I +3 primers**	
act-ctg	P1M1
acg-ctg	P2M1
acg-ctt	P2M2
agg-ctg	P4M1
agg-ctt	P4M2
agg-caa	P4M3
aag-ctt	P5M2
acc-ctg	P8M1

RAPD reactions consisted of 25 to 30 ng of lentil genomic DNA, 1 unit of *Taq* polymerase, 100 μM of each dNTP, 0.24 μM RAPD primer, and PCR reaction buffer [50 μM KCL, 10 μM Tris-HCL pH 8.3, 2.5 μM $MgCl_2$, and 0.1% (v/v) Triton X-100]. The following cycle was used 40 times to amplify DNA: 20 s at 94°C, 1 min at 36°C, 3 min ramp to 72°C, and 1 min at 72°C. The final elongation segment was held for 8 min at 72°C. Amplified PCR products were electrophoresed on 2% (w/v) agarose gels with 1× TBE buffer at 100 to 120 V for 3.0 to 3.5 h. The gels were stained with ethidium bromide and photographed under ultraviolet light.

For ISSR analysis, the PCR reaction mixture was the same as for RAPD analysis except that the concentration of the dNTPs was double (200 μM instead of 100 μM). The following PCR program was used to amplify the DNA samples: 94°C for 1 min, 50°C for 1 min, and at 72°C for 2 min. Final elongation step was held for 8 min at 72°C. PCR products were separated on a 4.5% (w/v) PAGE (polyacrylamide gel electrophoresis), then silver stained and scored for the presence or absence of bands. Nomenclature for the RAPD and ISSR marker loci was based on the primer name. For primers that amplified more than one polymorphic band, subscripts of 1, 2, 3, etc. (starting from highest to lowest molecular weight band) were assigned after the primer name.

For AFLP analysis, four *Eco*RI/*Mse*I (*Mse*I methylation insensitive) and eight *Pst*I/*Mse*I (*Pst*I is methylation sensitive) primer combinations were used (Table 1). The AFLP protocol of Vos et al. (1995) was employed based on established procedures (Barrett and Kidwell, 1998) and modified as described below.

Genomic DNA (about 500 ng in a 10-μL volume) was digested with 5U of either *EcoRI/Mse*I (Promega, Madison, WI, and Life Technologies, Inc., Gaithersburg, MD, respectively), or *Pst*I/*Mse*I (New England Biolabs, Inc., Beverly, MA) for 3 h at 37°C. in a digestion cocktail including 1× One-Phor-All (OPA) Buffer (Pharmacia), and 5 mg BSA for a total volume 50 μL. Adapters were ligated by means of 1U T4 DNA ligase (Promega), 1× T4 DNA ligase buffer, 5 pmol *Pst*I or *Eco*RI adaptor, and 50 pmol *Mse*I adaptor (Table 2). This ligation cocktail was added to the digested DNA (50 μL per sample from step1) and ligation proceeded at 25°C for 3 h. Adaptor ligation was completed by incubating the sample at 65°C for 20 min, and diluting products in TE buffer (10

Table 2. Oligonucleotides and their sequences used in AFLP analysis of lentil recombinant inbred lines.

Adapters and sequences		Primers and sequences	
PstI	**5′ ctc gta gac tgc gta cat gca cat ctg acg cat gt 5′**	***Pst*I + a**	**5′ gactgcgtacatgcag- aXX†**
EcoRI	**5′ ctc gta gac tgc gta cc cat ctg acg cat ggt taa 5′**	***Eco*RI + a**	**5′ gactgcgtaaccaattc- aYY‡**
MseI	**5′ gac gat gag tcc tga gta ctc agg act cat 5′**	***Mse*I + c**	**5′ gatgagtcctgagtaa- cZZ§**

† XX are null for plus 1 primers; CA, CC, or CT for plus 3 primers.
‡ YY are null for plus 1 primers; AC, AG, or CT for plus 3 primers.
§ ZZ are null for plus 1 primers; AA, AC, AG, AT, GA, GC, GG, GT, TA, or TG for plus 3 primers.

m*M* Tris, 1 m*M* EDTA pH 8.5). The preamplification cocktail consisted of 4 μL l0× PCR buffer (Promega), 50 ng *Eco*RI + A or *Pst*I + A, 50 ng *Mse*I + C, 0.2 m*M* each dNTP, 1.5 m*M* $MgCl_2$, and 0.8 U Taq polymerase (Promega). The PCR reaction (1–5 μL digested ligated DNA and preamplification cocktail for total volume of 38 μL) was run at 94°C for 2 min, 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min., followed by a final elongation step at 72°C for 5 min. PCR products were diluted 1 to 20 in TE. Selective amplification, cocktails were prepared as follows: (i) PCR cocktail (15 μL) for the *Eco*RI/*Mse*I selective amplification—2 μL 10× PCR buffer, 0.18 μL *Eco*RI+A-YY (Life Technologies, Gaithersburg, MD, AFLP kit #10483014), 4.5 μL *Msel*+C-ZZ + dNTPs (Life Tech. kit), 1.5 m*M* $MgCl_2$, 0.2 U Tag polymerase; and (ii) PCR cocktail (15 μL) for the *Pst*I/*Mse*I—2 μL 10× PCR buffer, 5 ng *Pst*I + A-XX, 30 ng *Mse*I + C-ZZ, 0.2 m*M* each dNTP, 1.5 m*M* $MgCl_2$, 0.2 U Taq polymerase. The selective PCR thermocycle profile consisted of 1 cycle at 94°C for 2 min; 13 cycles of 94°C for 30 s, 65°C for 30 s (0.7°C decrease in temperature per cycle for cycles 2 through 13) and 72°C for 1 min, 23 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and a final cycle of 72°C for 2 min. Selective amplification products were mixed with 7.5 μL loading buffer (98%, v/v, formamide, 10 m*M* EDTA, 0.15%, v/v, Bromophenol Blue, 0.15%, v/v, Xylene Cyanol) and 5 μL were loaded onto a 0.4-mm-thick 6% (w/v) denaturing polyacrylamide gel and resolved at constant power (80 W) in 1× TBE running buffer for 2.5 h. Bands were visualized by means of a commercial silver stain protocol (Promega, Madison, WI). Gels were scored visually while on the glass plate on a trans illuminator.

AFLP bands were designated as E_M_ for *Eco*RI/*Mse*I and P_M_ for *Pst*I /*Mse*I primer combinations (Table 1). For example, we denoted three nucleotide selective primers for *Eco*RI *act* as E1 and for *Mse*I *ctg* as M1 and E1M1 indicates the *act-ctg* combination. In the presence of more than one polymorphic band for any primer combination, subscripts of 1, 2, 3, etc. (starting from highest to lowest molecular weight band) were assigned after the primer name such as E1M1-1.

Linkage Mapping and QTL Analysis

Ninety-four RILs from the cross of WA8649090/Precoz were scored for 56 RAPD, 106 ISSR, 94 AFLP markers, and three morphological traits (plant height, fall growth habit, and leaflet size). Linkage analysis was performed by MAPMAKER 3.0 (Lander et al., 1987). Linkage criteria were set at LOD 3.0 with a recombination fraction of 0.30 cM. Kosambi mapping function was used to convert the recombination frequencies into genetic distances (Kosambi, 1944). Markers were ordered by multipoint analyses and ripple command was used to recheck the multipoint order of loci in each linkage group. Mapmaker linkage order results were reevaluated by comparing the results obtained from MapManager's REARRANGE option that rearranges the loci for specified groups.

Possible segregation distortion of marker loci from the expected Mendelian segregation ratio of 1:1 in a RIL population was determined using a Chi-square test. Groups of linked markers that were similarly distorted were used for linkage mapping and QTL analyses. Conversely, independent markers showing significant segregation distortion and markers with missing data were not included in QTL analyses to avoid bias and false results. Tightly linked or cosegregated markers were excluded because they have no effect on QTL detection (Kearsey and Pooni, 1996). A framework map comprised of 130 markers was used for QTL analyses.

QTL analysis was performed by Qgene 3.0 (Nelson, 1997) and MapManager QT 2.8 (Manly, 1998). Qgene was used for simple interval mapping, multiple regression, and to determine epistatic interactions. MapManager QT was used to check data quality and to confirm the results generated by other programs. Since this is the initial study for winter hardiness in lentil, a LOD score of 2.0 was chosen as the threshold for declaring putative QTL. QTL positions were determined by the peak LOD score. Multiple peaks within 30 cM were considered as a single QTL (Kearsey and Pooni, 1996). The percentage of the phenotypic variation (R^2) explained by the detected QTL was determined by multiple regression analysis using those markers explaining the peak response of individual QTL.

RESULTS

Informative results were not obtained due to mild winters and a lack of winter killing, at Pullman in 1997-1998, at Haymana and Sivas in 1998-1999, and complete killing at Sivas in 1999-2000 (Fig. 1). The distribution of winter survival scores at each environment showed deviations from normality; however, a normal distribution was observed when the data were averaged across three environments. Significant differences (Table 3) for winter survival in each environment year appeared to be due to contrasting winter conditions. Winter survival of the RILs was lower (5.3%) for the harsher conditions at Pullman than the moderate (49.7%) to mild (89.5%) winters at Haymana in 1997-1998 and 1998-1999, respectively.

Components of variance obtained by analysis of variance at each environment and for the average of the three environments were all highly significant ($P < 0.01$) (Table 3). The mean square due to location effect was the highest, and followed by mean square of RILs and the genotype × environment interaction component (Table 3). The field trials were exposed to contrasting winter conditions, which were confirmed by the variable survival scores for the RILs and the highly significant variance for locations. However, even for mild conditions, significant segregation for winter survival among lines was observed (Table 3). The contrasting environmental conditions likely contributed to the significant RIL × environment interaction.

Monitoring winter injury at monthly periods during the snowless winter of 1998-1999 at Pullman depicts a cumulative effect of winter stress factors on winter

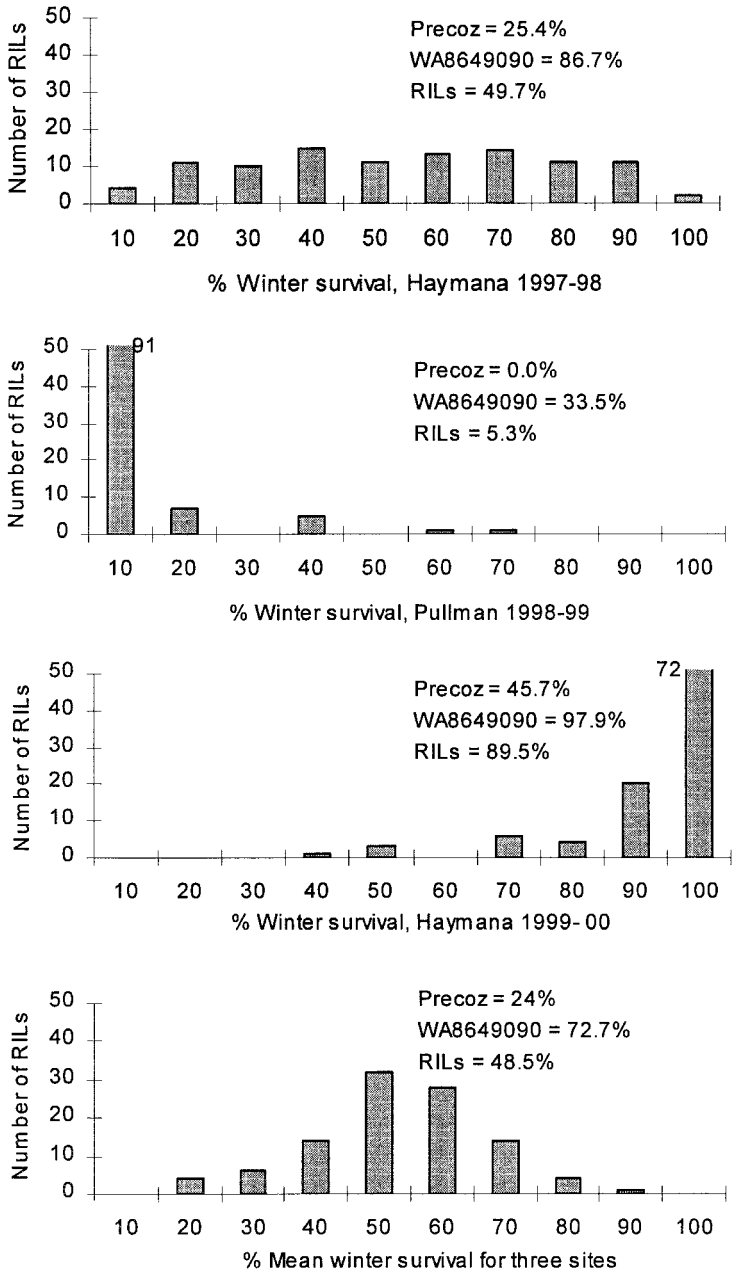

Fig. 1. Frequency distributions of winter survival scores at Haymana, Turkey in 1997-1998 and 1999-2000, at Pullman, WA, USA, in 1998-1999 and mean winter survival for three sites over three years.

survival. The January scores for winter injury were obtained after 8 d of cold (minimum air and soil temperatures were –19.5°C and −10.5°C, respectively) without snow cover. Seedlings had four to seven nodes and were 40 to 60 mm tall. The nonhardy Brewer check was completely withered and wilted and nonhardy parent Precoz had winter injury scores of 96.7% suggesting no possibility of recovery for these genotypes, whereas the winter hardy parent WA8649090 had only 10.0% winter injury. About one third of the RILs had winter injury scores above 50.0%, and 14 RILs had winter injury scores similar to the non-hardy parent.

The February scoring was made after a second cold period (minimum air and soil temperatures were –12.1°C and –5.8°C, respectively) without snow cover. No change was observed for winter injury to the hardy parent while the nonhardy parent Precoz and the Brewer check were completely withered (Fig. 2). Approximately half of the RILs had injury scores above 50% and most of the remaining RILs had winter injury scores above 90%.

The March scoring occurred after a short period of moderate cold exposure (minimum air and soil temperatures were –6.5°C, and –3°C, respectively). Injury to the winter hardy parent did not change and remained at about 10% (Fig. 2). Interestingly, average winter injury scores for the RILs appeared lower than February scores. The decreased injury scores appeared to result from regrowth and recovery of the plants during warm temperatures in late February.

The final April scoring followed a warming trend

Table 3. Analysis of variance of lentil winter survival at Haymana, Turkey, in 1997-1998, Pullman, WA, USA, in 1998-1999 and Haymana, Turkey, in 1999-2000, and the combined analysis across environments.

Source	DF	MS	F	P
		1997–1998		
Rep	2	1 651.6	4.41	0.0134
RIL	102	1 774.3	4.74	0.0001
Error	204	374.6		
		1998–1999		
Rep	2	65.4	1.38	0.2530
RIL	107	453.6	9.59	0.0001
Error	214	47.3	9.44	
		1999–2000		
Rep	2	707.6	6.82	0.0014
RIL	107	590.6	5.69	0.0001
Error	214	103.8		
		Across environments		
Loc	2	576 499.8	3 218.8	0.0001
Rep	2	86.2	0.48	0.6179
RIL	106	1 572.9	8.78	0.0001
RIL×loc	210	628.1	3.51	0.0001
Error	636	179.1		

(average air temperatures were 2°C and 5°C in February and March, respectively). An increase of about 50% winter injury to the hardy parent was observed. Average winter injury for the RILs was 82.9% indicating severe damage with little recovery. Increased winter injury scores from March to April likely the result from the cumulative effects of winter conditions, particularly the cold periods and freeze–thaw cycles, and sensitivity to cold temperatures during the dehardening process in the spring. In April, when the final survival scores were determined based on plant stand counts, the hardy parent had only 33.5% survival and the RILs had a mean survival of only 5.3%. We observed soil borne diseases in the hardy parent during plant regrowth in spring and it appeared that disease susceptibility may have prevented plant recovery and increased winter killing.

Linkage Mapping Results

The RILs were genotyped for 56 RAPDs, 106 ISSRs, and 94 AFLPs. Of these 256 markers, 84 were excluded from the QTL analysis because of lack of linkage, incomplete data, or distorted segregation. Groups with distorted segregation in linkage groups 3 (P5M2-2 and ubc541-1), 7 (ubc841-8, cs31-1, cs31-3 and ubc822-7), and 8 (ubc449-2, ubc809-2, P4M3-4, cs54-2 and ubc809-8) were included because they do not affect linkage and QTL analyses (Kearsey and Pooni, 1996). A total of 175 markers (complete marker list available on request) were used to construct a linkage map with nine linkage groups (Fig. 3). For QTL analyses, a framework of 130 markers covering 1192 cM of the lentil genome was used. Average distance between markers was 9.1 cM and ranged from 0.3 to 21.1 cM.

We could not compare our linkage map with the previously published linkage maps of lentil (Muehlbauer et al., 1989; Tahir, 1990; Eujayl et al., 1998) because no markers were common. There was a lack of polymorphism for isozyme markers in our mapping population and a lack of segregation for previously mapped morphological traits. Also, RFLP markers were not used

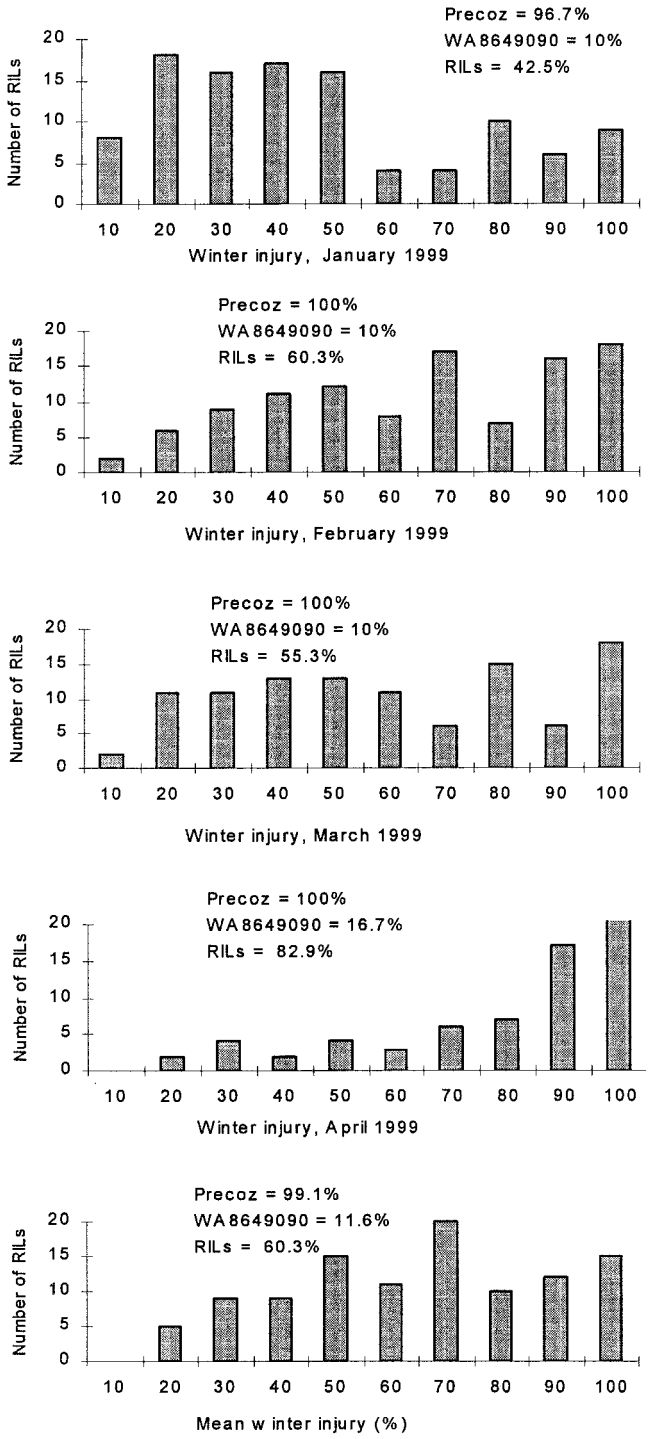

Fig. 2. Frequency distributions for winter injury during the winter of 1998-1999 at WA, USA.

and the RAPD primers and selective primer combinations for AFLPs were different from the published linkage maps. The ISSRs were unique to this linkage map. ISSR primers generated numerous polymorphisms (average eight polymorphic bands per primer) some of which were codominant markers.

QTL Results

Five independent QTL were detected for winter survival (Table 4). One QTL on linkage group 4 and two

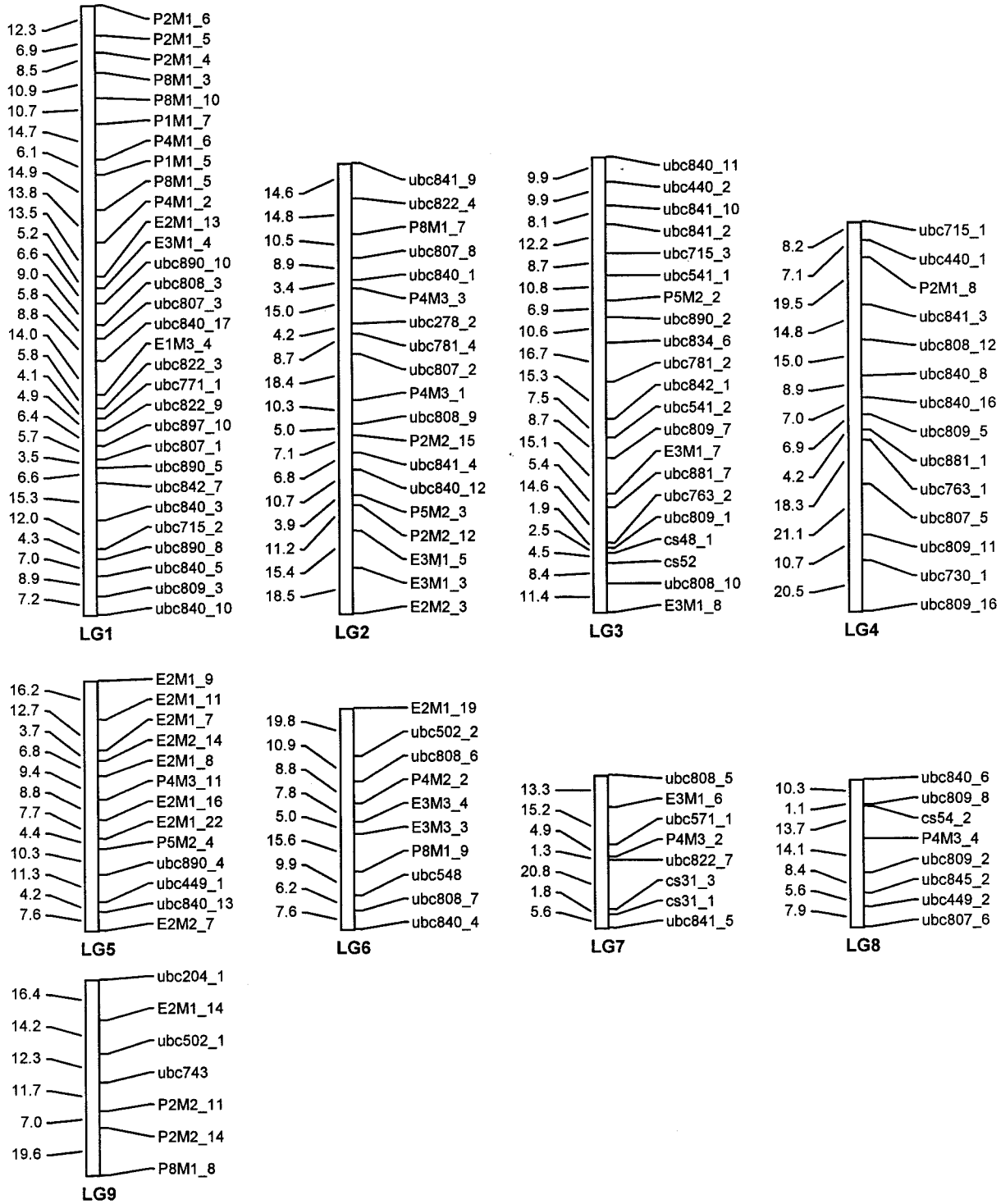

Fig. 3. Genetic linkage map of lentil based on AFLP, RAPD, and ISSR marker loci.

QTL on linkage groups 3 and 6, respectively, were detected for winter survival at Haymana in 1997-1998 (Table 4). Together these QTL explained 33.4% of the total phenotypic variation for winter survival. Under harsh winter conditions at Pullman, where there was 95% mortality, one QTL was detected on linkage group 4. In the presence of mild winter conditions at Haymana in 1999-2000, three putative QTL were detected, two on linkage group 1 and one on linkage group 4. Together the QTL explained 22.9% of the phenotypic variation. The QTL located on linkage group 4 was common to all environments and years, but the effect and position differed across environments (Table 4). When winter survival data from all sites were combined and subjected to QTL analysis, the two QTL on linkage groups 4 and 6, were detected.

Four QTL were identified for winter injury scores at (Fig. 4). Three QTL were located on linkage group 1

Table 4. Putative QTL for lentil winter survival at Haymana, Turkey, in 1997-1998, at Pullman, WA, USA, in 1998-1999, and at Haymana, Turkey, in 1999-2000 and QTL detected for winter injury at Pullman, WA, USA, in 1998-1999.

Location	Linkage group	QTL position (cM)†	LOD	R^2 (%)	P	Additive effect
Haymana 1997-1998	3	28	2.3	10.9	0.0012	8.4
	4	118	7.3	28.8	0.0000	15.3
	6	80	3.2	17.7	0.0001	11.9
				Total R^2 = 33.4		
Pullman 1998-1999	4	110	2.5	11.5	0.0008	4.7
Haymana 1999-2000	1	38	2.3	9.5	0.0025	4.2
	1	146	2.2	10.1	0.0017	4.2
	4	132	2.0	9.5	0.0005	4.2
				Total R^2 = 22.9		
Combined	4	116	6.9	28.8	0.0002	7.7
	6	80	3.1	14.2	0.0002	6.2
				Total R^2 = 31.5		
		QTL for mean winter injury detected at Pullman in 1998-1999				
	1	39	5.4	23.2	0.0000	11.3
	1	91	2.7	13.4	0.0005	8.7
	1	129	2.2	10.9	0.0015	7.7
	4	112	2.3	10.9	0.0013	7.6
				Total R^2 = 42.7		

† QTL positions were determined from the bottom of each linkage group.

and one was located on linkage group 4. The four QTL accounted for 42.7% of the variation for winter injury. Three of the QTL conditioning winter injury were located in genomic regions where QTL for winter survival were located (Fig. 4). The QTL on linkage group 1 at position 39 and 129 cM were also detected at Haymana (1999-2000), and the QTL on linkage group 4 was likely the same QTL at all locations.

DISCUSSION

The two parents, Precoz and WA8649090, differed widely for winter survival. The hardy parent WA8649090 survived well at Haymana in under moderate winter conditions. Poor survival of WA8649090 at the Pullman location, in addition to harsh winter conditions, may have been due to disease susceptibility (apparently lack of resistance to soil borne fungi, perhaps *Pythium*). Root rot disease is suspected because of the increasing injury incurred during the warmer months of February and March. Perhaps WA8649090 suffered damage to the roots during the winter, which did not manifest into visible injury until April when damaged roots would be expected to be susceptible to a wide range of soil-borne pathogens. Winter injury contributed to weak plant regrowth and survival. On the basis of these observations and those of Murray et al. (1988), resistance to soil borne fungi should be considered for successful selection of winter hardy grain legumes.

The first three scores for winter injury were assumed to be caused by low temperatures because no snow cover was on the field. Therefore, winter injury scores were considered a measure of cold tolerance, and the inheritance of cold tolerance based on frequency distribution (Fig. 2) pattern and QTL analysis indicated multiple gene control. Our results support previously published reports in other crops that winter hardiness is a complex trait (Hayes et al., 1993; Grafius, 1981; Blum, 1988). The complexity can be due to effects at more than one locus, and the interactions of these loci with environment.

In different test winters, specific components may be critical for survival (Palta et al., 1997). For example, winter survival in some years was affected more by fluctuating temperatures and fungal diseases than by exposure to lethal freezing temperatures (Chun et al., 1998). In our experiments at Pullman under the snowless winter of 1998-1999, we observed that prolonged cold periods, freeze–thaw cycles, and disease susceptibility were the major factors for winter killing. Prolonged cold was the main factor for winter injury, freeze–thaw cycles caused more injury and injured plants were highly vulnerable to disease infection. In other studies, different results were reported for the causes of winter injury. Salmon (1932; cited in Grafius, 1981) reported the primary causes of winter injury as heaving, smothering, physiological drought, and freezing of the plant tissue.

We have observed that duration and frequency of low temperature was the cause of poor survival rather than low temperature itself as reported for other crops (Gusta et al., 1997; Taylor and Olsen, 1985). For example, in the 1997-1998 field trial at Pullman, minimum air temperature was –16.5°C but plants were covered with snow most of the time and the duration of the low temperatures was less than 1 d. Also, throughout most of the winter season, the plants were covered with snow or temperatures were not much below 0°C. Therefore, no winterkill was observed that year and the nonhardy parent Precoz and nonhardy check Brewer had 100% survival.

Acclimation to low temperatures is a cumulative process that can be reversible depending on changes in temperature. When we scored for winter injury in March 1999 at Pullman, the average air temperature was above 10°C. These warm temperatures could trigger dehardening and increase susceptibility to cold. The threshold temperature for acclimation of winter cereals was reported to be about 10°C (Olien, 1967). The threshold temperature for acclimation of lentil is not known; however, significant differences in temperature requirements for dehardening are not expected. Any deharden-

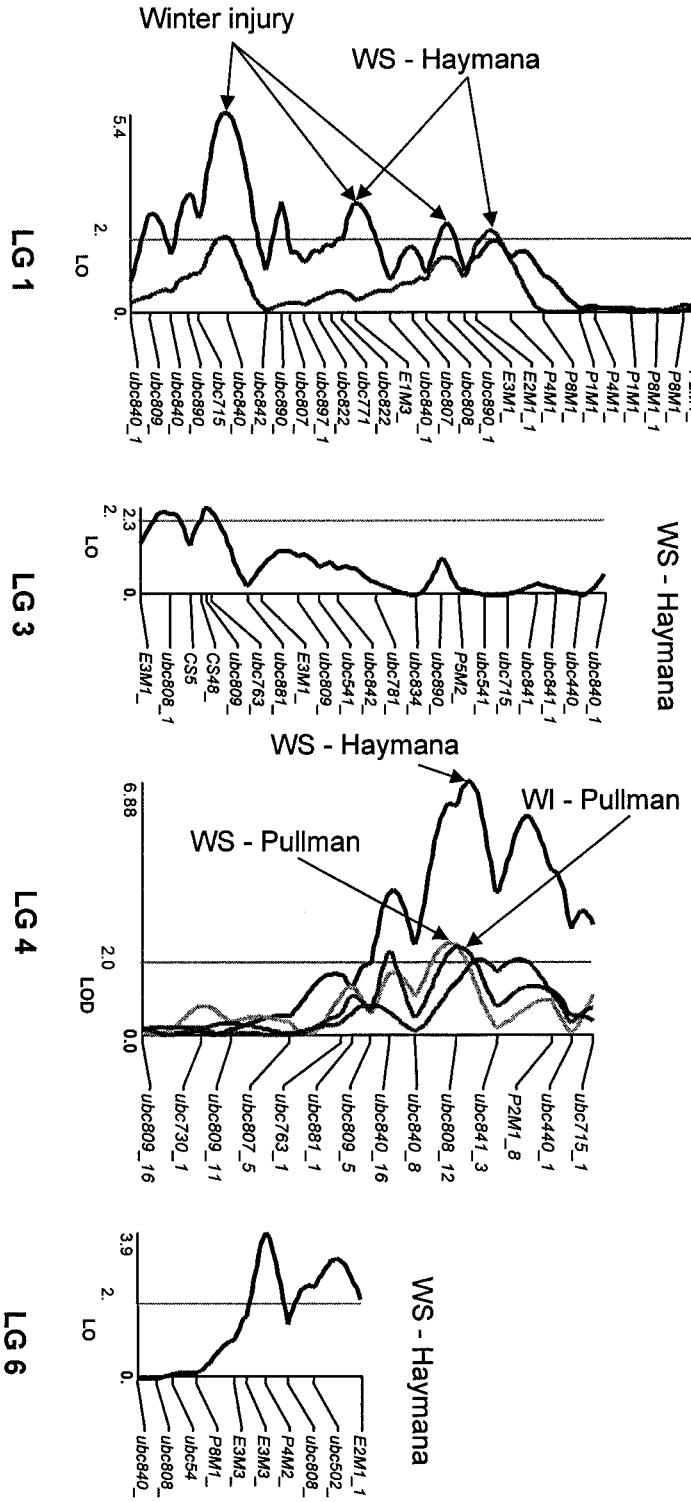

Fig. 4. QTLs detected for winter survival at Haymana, Turkey, in 1997-1998, at Pullman, WA, USA, in 1998-1999, and at Haymana, Turkey, in 1999-2000 and QTLs detected for mean winter injury at Pullman, WA, USA, during the winter of 1998-1999. WS = winter survival, WI = winter injury, LG = linkage group.

ing process will make the plants, which already were damaged by early cold periods in January and February, more vulnerable and sensitive to frost damage late in the spring.

Although five QTL were detected for winter survival, only one, the QTL on linkage group 4 for winter survival expressed across all environments. QTL that show consistency in expression across environments, even in diverse environments, are desirable for marker assisted selection programs (Veldboom and Lee, 1996). Although somewhat consistent, slight differences in expression of the QTL on linkage group 4 might be due

to the sensitivity of the QTL to the contrasting winter stresses encountered in each environment. Presence of different QTL for winter survival from the same location (Haymana) in different years supports the premise that winter stress factors had a greater influence on QTL detection than location effects.

Significant differences between RILs for winter injury suggest the G×E is a result of magnitude differences in response, not changes in the ranking of responses; thus, an individual environment may be suitable for detection of superior genotypes. Environments with moderate to severe winter conditions will provide the best opportunities to identify superior genotypes.

We have identified candidate molecular markers for winter survival on the basis of QTL analyses that could be used in marker assisted selection programs. ISSR marker ubc808-12 (linkage group 4) was consistent across environments. Another ISSR marker ubc840-3 was associated with winter injury at Pullman in 1998-1999 and winter survival at Haymana in 1999-00. The markers for the identified QTL should be evaluated for their effectiveness in marker assisted selection for winter hardiness in a divergent group of lentil crosses. Successful use of marker assisted selection will accelerate selection for winter hardiness particularly when faced with the prospect of mild or extremely severe nontest winters.

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